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Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C: Prediction of a cofactor to activated protein C

(protein S/thrombosis/blood coagulation/anticoagulation)

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ABSTRACT **Although patients with thromboembolic disease frequently have family histories of thrombosis, well-defined defects such as inherited deficiencies of anticoagulant proteins are found only in a minority of cases. Based on the hypothesis that a poor anticoagulant response to activated protein C (APC) would predispose to thrombosis, a set of new coagulation assays was developed that measure the anticoagulant response in plasma to APC. A middle-aged man with a history of multiple thrombotic events was identified. The addition of APC to his plasma did not result in a normal anticoagulant response as measured by prolongation of clotting time in an activated partial thromboplastin time (APTT) assay. Four of the proband's relatives had medical histories of multiple thrombotic events, and they and several other family members responded poorly to APC in the APTT-based assay. Subnormal anticoagulant responses to APC were also found in factor IXa- and Xa-based assays. Several possible mechanisms for the observed phenomenon were ruled out, such as functional protein S deficiency, a protein C-inhibitory antibody, or a fast-acting protease inhibitor against APC. Moreover, restriction fragment-length polymorphism analysis excluded possible linkage of the underlying molecular defect to factor VIII and von Willebrand factor genes. We now describe a previously unrecognized mechanism for familial thromboembolic disease that is characterized by poor anticoagulant response to APC. This would appear to be explained best by a hypothesized inherited deficiency of a previously unrecognized cofactor to APC. As we have identified two additional, unrelated cases with thrombosis and inherited poor anticoagulant response to APC, this may constitute an important cause for familial thrombophilia.**

A physiologically important anticoagulant system has been elucidated in recent years. A key component in the system is protein C, a vitamin K-dependent plasma protein which, after its activation on endothelial cells by the thrombin–thrombomodulin complex, selectively degrades coagulation factors Va and VIIIa (1–3). Another vitamin K-dependent plasma protein, protein S, functions as a cofactor to activated protein C (APC). An increased risk of venous thrombosis in young and middle-aged adults is associated with heterozygous deficiency of either protein C or protein S.

Patients with thromboembolic disease occurring at a relatively young age frequently have family histories of thrombosis, suggesting genetic factors to be involved. However, heterozygous deficiencies of anticoagulant proteins can only be found in a minority of cases (4). This suggests that there are genetic defects predisposing for thrombosis yet to be identified.

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We have now identified a previously unrecognized mechanism for familial thromboembolic disease that is characterized by a poor anticoagulant response to APC. Based on experimental data presented in this paper, it is hypothesized that this is due to a deficiency of a previously unknown APC cofactor.

MATERIALS AND METHODS

Case Report. The proband is a male born in 1942. At the age of 19 years, he had his first episode of deep venous thrombosis in one leg. After this, he was healthy and free of thrombosis for almost 20 years. Between 1980 and 1987 he had multiple episodes of deep venous thrombosis, at least once a year. The thrombotic events were treated with vitamin K antagonists for periods of up to 3 months. The presence of a thrombus was verified with phlebography on at least two occasions. The proband has developed a postthrombotic syndrome in his legs but has no other disorders. Several members of the proband's family have similar histories of multiple episodes of deep venous thrombosis (Fig. 1). His older brother by 10 years (III-2) has had deep venous thrombosis (in the legs) on several occasions, most of them occurring between the ages of 45 and 50. Also his uncle (II-7) and aunt (II-5) have both had multiple episodes of thrombosis. A younger relative (IV-2) had clinically suspected deep venous thrombosis during her third pregnancy, but phlebography failed technically. The proband's father, who had no history of thrombosis, is deceased. Nineteen of the family members (all living members of generations II–IV) were available for testing. Two additional, unrelated cases with thrombophilia and inherited poor response to APC were identified; their medical histories are briefly described in the legend to Fig. 6.

Analytical Methods. Blood sampling and routine coagulation methods were performed as described (4). The plasma concentrations of protein S and protein C were determined with immunochemical methods (4) and with commercially available functional assays: Coatest protein C (Chromogenix, Mölndal, Sweden) and Staclot protein S (Stago, Asnières, France).

An activated partial thromboplasmin time (APTT)-based method was developed to determine the anticoagulant response in patient plasma to added purified APC. In this method, which will be referred to as the APC-APTT assay, the APC-mediated prolongation of clotting time was measured in duplicate as follows: 0.1 ml of plasma was incubated with 0.1 ml of APTT reagent [APTT-automated or Platelin

Abbreviations: APC, activated protein C; APTT, activated partial thromboplastin time; APC-APTT method, APTT-based method measuring the anticoagulant response to APC; BSA, bovine serum albumin.

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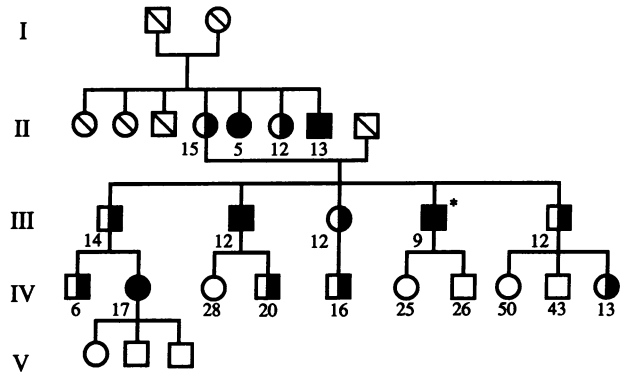

FIG. 1. Five generation pedigree of a family with thromboembolic disease. The proband (III-4) is indicated by an asterisk. Circles denote females, squares denote males, and diagonal lines denote deceased family members. Half-filled symbols indicate subjects with APC-dependent prolongation of clotting time below the fifth percentile of controls (<23 sec), whereas filled symbols denote subjects with both a history of thrombosis and a poor anticoagulant response to APC. The APC-dependent prolongation of clotting time, as measured by the APC-APTT method (each value represents the mean of two to four measurements performed on different days with automated APTT as reagent), is given below each symbol.

LS (Organon Teknika) as indicated in the figure legends] for 5 min at 37°C before coagulation was initiated by the addition of 0.1 ml of an APC/$CaCl_2$ mixture [if not indicated otherwise, 20 nM human APC in 10 mM Tris·HCl/0.05 M NaCl/30 mM $CaCl_2$, pH 7.5/0.1% bovine serum albumin (BSA)], the coagulation time being recorded with an MLA 900C apparatus. Each plasma sample was also analyzed in parallel without the addition of APC to the $CaCl_2$ solution, and the APC-dependent prolongation of clotting time was calculated. In a separate set of experiments, human APC was exchanged for its bovine counterpart. The effect of bovine APC (5–160 nM in the APC/$CaCl_2$ mixture) was tested in both the presence and absence of bovine protein S (the final protein S concentration in the assay was 44 nM). The human and bovine APC and protein S were prepared as described (5).

Factor IXa and Xa-based assays were also used to analyze the anticoagulant response to human APC. In these assays, 0.1 ml of plasma was mixed with 0.1 ml of bovine factor Xa (0.5 nM) [or bovine factor IXa (12 nM)] and 0.1 ml of rabbit brain cephalin (Sigma). The reagents were diluted in 50 mM Tris·HCl/0.15 M NaCl, pH 7.5, containing 0.1% BSA. Coagulation was initiated with 0.1 ml of an APC/$CaCl_2$ mixture (10 mM Tris·HCl/50 mM NaCl/30 mM $CaCl_2$, pH 7.5/0.5% BSA/10–50 nM human APC), and the clotting time was recorded. Each sample was also tested in parallel without the addition of APC in the $CaCl_2$ solution. In the absence of APC, the clotting times were between 30 and 50 sec. Purified bovine factors Xa and IXa were gifts of Egon Persson and Jan Astermark (Department of Clinical Chemistry, Malmö, Sweden), respectively.

To test the rate of inhibition of APC in plasma, 300 μl of plasma was mixed with 200 μl of bovine APC (80 nM in 50 mM Tris·HCl/0.1 M NaCl, pH 7.4/0.1% BSA) and incubated at 37°C. At intervals, 30-μl aliquots were drawn, mixed with 300 μl of 0.3 mM S-2366 (Chromogenix), and incubated at 37°C for 10 min. Acetic acid (50 μl of a 50% solution) was added to stop the reaction, and the absorbance was measured at 405 nm. The concentration of active APC was calculated from a standard curve, which was constructed by using purified bovine APC. APC added to a 6% BSA solution remained stable throughout the experiment.

Absorption of IgG, IgA, and IgM from patient plasma was performed as described in detail (6).

DNA Polymorphism Analysis. DNA was isolated from EDTA blood (7). A *Bcl* I polymorphism in the factor VIII gene was analyzed as described (8). *Hph* I and *Bst*EII polymorphisms in exon 28 of the von Willebrand factor gene were analyzed as reported (9, 10). The different genotypes of the *Hph* I polymorphism were designated h^+ and h^-, and the *Bst*EII alleles were designated + and −. In addition, a variable-number tandem repeat (VNTR) analysis of a polymorphic site in the von Willebrand factor gene was performed as described by Peake and coworkers (11).

Statistical Analysis. The Mann–Whitney *U* test was used for comparing populations.

RESULTS

The proband had a history of multiple episodes of deep venous thrombosis, and his family history revealed a high incidence of thrombosis among his relatives, suggesting an inherited cause of the disease (Fig. 1). Values for antithrombin III, protein C, protein S, plasminogen, fibrinogen, thrombin/reptilase times, and routine coagulation variables were normal, and there was no indication of the presence of lupus anticoagulants (data not shown).

New assays were developed that measured the anticoagulant response in patient plasma to added APC. In the APC-APTT assay, the anticoagulant response of proband's plasma to APC was consistently much smaller than that of control plasma (Fig. 2). Fourteen of the 19 tested family members manifested APC-dependent prolongation of clotting time below the fifth percentile of controls, suggesting the underlying biochemical defect to be inherited (Figs. 1 and 3). The range of values of controls was wide and the distribution was skewed but became essentially normal after logarithmic transformation.

The poor anticoagulant response to APC could be due to any of the following mechanisms: (*i*) an autoantibody to protein C, (*ii*) a fast-acting protease inhibitor to APC, (*iii*) protein S deficiency, (*iv*) mutations in the genes for factors VIII or V (in or close to the regions encoding the APC-cleavage sites), and (*v*) a previously unrecognized mechanism. These possibilities were investigated.

As the APC resistance was inherited, it was unlikely to be due to an inhibitor of immunoglobulin type. However, to exclude the possibility, the proband's plasma was again tested in the APC-APTT assay when depleted first of IgG, then IgA and finally IgM. Despite the complete removal of the respective immunoglobulin, the proband's plasma still responded poorly to APC, as compared with control plasma treated in the same way (data not shown), arguing against an

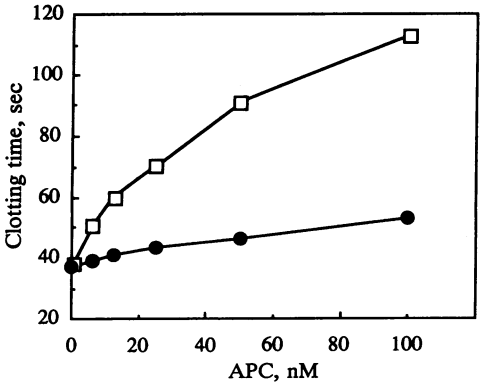

FIG. 2. Poor anticoagulant response to APC in the proband's plasma. Increasing concentrations of human APC (final concentrations in assay) were included in the APC-APTT method, and the clotting time is recorded. ●, Proband's plasma; □, control plasma. Each point represents the mean of duplicate values.

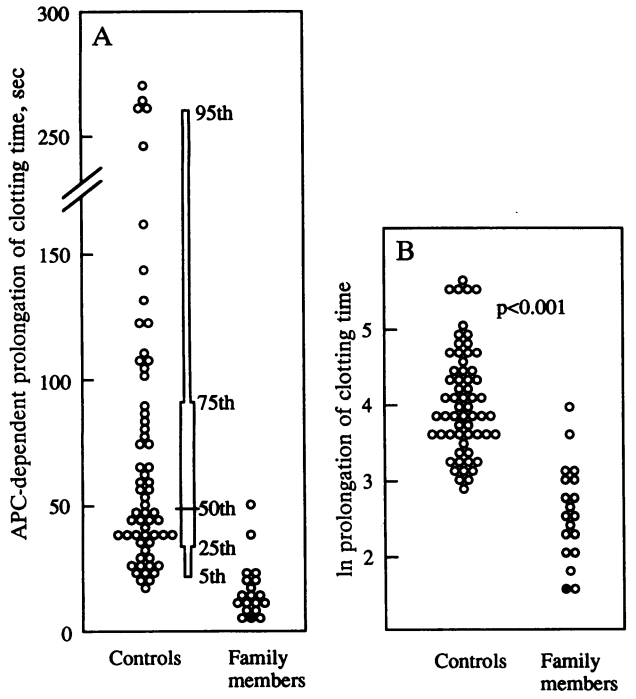

FIG. 3. Anticoagulant response to APC of family members and controls. Family members and controls were analyzed with the APC-APTT method (automated APTT was used as reagent in these measurements). The APC-dependent prolongation of clotting time (mean of duplicate measurements) is plotted. Each individual is represented by a circle, the filled circle denoting the proband. The range in the control group was 17–269 sec. The 5th, 25th, 50th, 75th, and 95th percentiles of controls are indicated. (*A*) Linear representation of the data. (*B*) Graphic presentation of the data after logarithmic transformation (ln, natural logarithm).

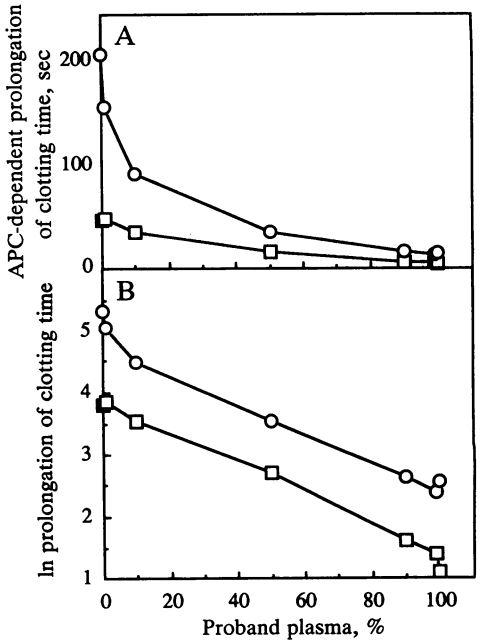

FIG. 4. Anticoagulant response to APC of mixtures of proband and normal plasma. Normal and proband plasma were mixed as indicated and analyzed with the APC-APTT method with two different final APC concentrations: 80 nM (○) and 8 nM (□). The APC-dependent prolongation is plotted on both a linear (*A*) and natural logarithmic (*B*) scale.

APC autoantibody. The presence of a fast-acting protease inhibitor reacting with APC could be excluded, as APC was inhibited at a normal rate in the proband's plasma ($t_{1/2}$ = 20 min). To further elucidate the possibility of an inhibitor causing the apparent APC resistance, mixtures of control and proband plasma were tested in the APC-APTT method by using two different APC concentrations (Fig. 4). When plotted against the percentage of proband plasma, the two curves were exponential; however, after logarithmic transformation of the data, they were essentially linear and parallel. These results argued against the presence in the proband's plasma of an APC inhibitor.

Inherited functional protein S deficiency was ruled out by the normal values obtained when testing the proband's plasma with a functional protein S assay. Moreover, the addition of purified human protein S to the proband's plasma did not correct the poor response to APC (data not shown). It was also observed that bovine APC, added with or without bovine protein S, was considerably less efficient in prolonging the clotting time of proband plasma than that of control plasma (data not shown). Because the APC–protein S interaction is species-specific (3), the possibility of the poor APC response being due to a functional protein S deficiency was ruled out.

To further elucidate the nature of the inherited defect, the anticoagulant response to APC in factor IXa- and factor Xa-based clotting assays was investigated (Fig. 5). The factor IXa-based assay was almost as efficient as the APC-APTT assay in distinguishing family members from normals. The family members manifested significantly poorer anticoagulant response to APC than controls even in the factor Xa-based assay, although the difference between controls and family members was less pronounced than in the other two assays. The correlation coefficient between the APTT- and factor IXa-based assays was 0.71 ($P < 0.001$), that between the APTT- and factor Xa-based assays was 0.41 ($P = 0.004$), and that between the factor IXa- and Xa-based assays was 0.68 ($P < 0.001$) (results of family members and controls were combined in these calculations). The combined results argue against the possibility of the poor APC response being due to a factor V mutation. Moreover, the subnormal response to APC in the factor Xa-based assay among family members made the possibility of a mutation in the factor VIII gene less likely.

A linkage study with markers for DNA polymorphisms in factor VIII and von Willebrand factor genes was performed to investigate whether the inherited poor APC response was due to mutations in these two genes. The proband's mother (II-4) was heterozygous for a factor VIII gene *Bcl* I polymorphism; two sons (II-1 and II-2) had inherited the allele denoted 99/43, whereas the other two sons (II-4 and II-5) had inherited the other allele (designated 142). As all four sons demonstrated poor APC response, it was concluded that the underlying defect was unrelated to the factor VIII gene. Markers for polymorphic sites in the von Willebrand factor gene showed three individuals in generation III (III-1, III-3, and III-4) to have inherited one of the mother's two von Willebrand factor alleles (designated 12,h^+,−), whereas III-2 and III-5 had inherited the other, thus precluding linkage to the von Willebrand factor gene.

Two additional cases with thrombophilia and inherited poor anticoagulant response to APC were identified (Fig. 6). Both had normal values for antithrombin III, protein C, protein S, plasminogen, and thrombin/reptilase times, and routine coagulation variables were normal.

DISCUSSION

The new coagulation-based assays that we developed measure anticoagulant response in plasma to added APC. To our knowledge, this type of assay has not been used before to

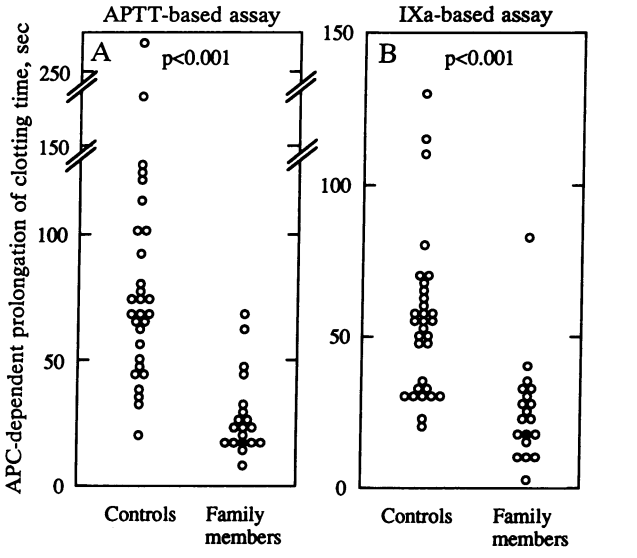

FIG. 5. Comparison of anticoagulant response to APC in APTT-, factor IXa-, and Xa-based assays. The anticoagulant responses to APC of family members and controls were measured with three assays as indicated, and the APC-dependent prolongation of clotting time was plotted. Each individual is represented by a circle and the proband, by a filled symbol. Platelin LS was used as the APTT reagent in this experiment.

investigate patients with thromboembolism. So far, assays based on measurements of the anticoagulant activity of APC have been devised to identify individuals with protein C or protein S deficiency. Usually these assays include protein C- or protein S-deficient plasma, and the plasma to be tested contributes the missing factor. The assays we have developed were designed to measure the anticoagulant response in plasma to added purified APC rather than proteins C or S, and they were based on the hypothesis that a poor response to APC would predispose to thrombosis. A poor anticoagulant response to APC could be due to any of a number of mechanisms, some known to exist and others hypothetical. They include the presence of autoantibodies against protein C, a fast-acting protease inhibitor to APC, protein S deficiency, mutations in the APC-cleavage sites of factors VIII or V, and finally involvement of previously unrecognized mechanisms and factors.

An autoantibody to protein C in a patient with thrombosis has been described (12). However, the poor anticoagulant response to APC in the proband's plasma was not caused by an antibody. The underlying cause was instead related to an inherited molecular defect. A 50% transmission of the poor anticoagulant response to APC between generations III and IV (Fig. 1) suggested an autosomal dominant mode of inheritance, which was also compatible with results of the other two families (Fig. 6). However, it is noteworthy that all tested members of generations II and III in the first family (Fig. 1) manifested poor anticoagulant responses to APC, and the molecular background for this remains to be elucidated.

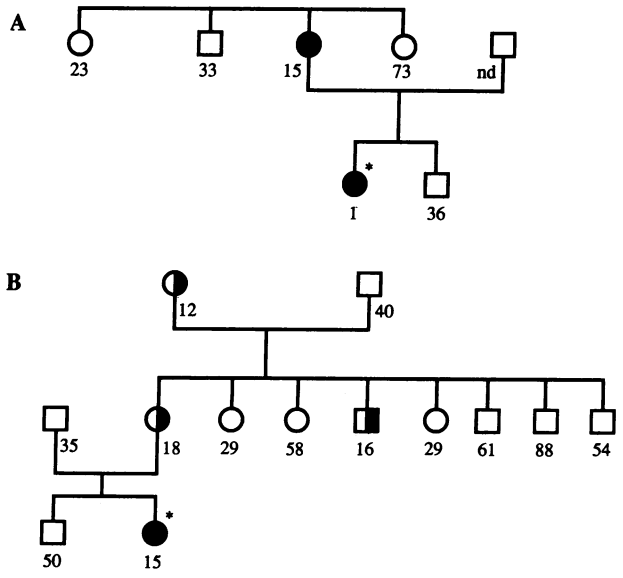

FIG. 6. Pedigrees of two families with thrombophilia and poor anticoagulant response to APC. Symbols are used as described in the legend to Fig. 1. In both families, the thromboembolic events occurred during pregnancies or in the postpartum period. The APC-dependent prolongation of clotting time is given below each symbol. nd, Not determined.

Protein S deficiency was excluded, as normal protein S levels were found in both immunological and functional assays. Moreover, addition of purified human protein S to the proband's plasma did not correct the poor anticoagulant response. In fact, several lines of experimental results suggested that the new APC-APTT assay used here does not detect protein S deficiency. Thus, using the APC-APTT method, several patients with known inherited protein S deficiency manifested anticoagulant response to APC within the normal range (B.D., unpublished observation). Moreover, protein S immunodepletion of normal plasma results in only an ≈50% reduction of the APC-dependent prolongation of clotting time in an APTT assay (13). Thus, it is concluded that poor anticoagulant response to APC in the APC-APTT assay is unrelated to protein S deficiency.

When added to normal plasma, APC is slowly inhibited by protease inhibitors (14). It has been reported that a mutation in the α1-antitrypsin (protease inhibitor) gene *PI* resulted in an efficient thrombin inhibitor, which caused a severe bleeding disorder in a young boy (15). Although unlikely, it was theoretically possible that the resistance to APC in the proband could be due to a mutation in the gene of one of the plasma protease inhibitors, creating an efficient APC inhibitor. This possibility was excluded, however, as APC, when added to proband's plasma, was inhibited at a normal rate. Moreover, the normal values for protein C in proband plasma, as measured by an amidolytic assay, precluded an inhibitor blocking the active site of APC.

APC exerts its anticoagulant effect through proteolytic degradation of factors VIIIa and Va (1–3). It is well known that mutation in the factor VIII gene (located on the X chromosome) is associated with hemophilia (2). It is hypothetically possible that mutations in or close to the APC cleavage sites could yield factor VIII molecules that are resistant to APC. Many of the present observations were compatible with the presence in the proband's plasma of such a modified factor VIII molecule. However, the possibility was excluded by DNA linkage analysis, which showed the proband and his affected brothers to have different factor

VIII genotypes. Factor VIII in plasma circulates in a complex with von Willebrand factor (2). The possibility that the poor APC response was linked to the von Willebrand factor gene was excluded on basis of DNA polymorphism analysis. Although linkage analysis to the factor V gene was not performed, a mutation in the factor V gene was unlikely because the poor APC response was less pronounced in the factor Xa assay (which only reflected factor Va degradation) than in the APTT- and factor IXa-based assays (which were sensitive to inhibition of both factors VIIIa and Va). The poor anticoagulant response to APC in the APC-APTT assay and in the factor IXa-based assay appears to be due to a combination of subnormal APC effects in the degradation of both factors VIIIa and Va.

The inherited poor anticoagulant response to APC described here cannot be explained by the currently accepted scheme of the protein C anticoagulant system. On the basis of results presented in this paper, it would appear to be reasonable to hypothesize the presence in normal plasma of a previously unrecognized cofactor to APC. The poor anticoagulant response to APC found in the proband and many of his relatives appears to be best explained by an inherited deficiency of such a cofactor. Although elucidation of the true nature of the proposed APC cofactor must await its isolation, it is already possible to draw some conclusions as to its mode of action. First, as the anticoagulant response to APC of the proband's plasma is smaller than that of protein S immunodepleted plasma, the putative APC cofactor would appear to function independently of protein S. On the other hand, the anticoagulant effect of protein S may depend on the presence of the putative APC cofactor because the proband's plasma, which contains normal concentrations of protein S, responded so poorly to added APC. Moreover, the addition of purified protein S did not correct the defective APC response. Whether the putative APC cofactor works in synergy with protein S will be possible to investigate once it is isolated. In this context it is interesting to note that Walker (16) reported in 1986 the presence in bovine plasma of a protein S binding protein that enhanced the anticoagulant activity of protein S. Unlike the now hypothesized factor, this protein did not work as an APC cofactor in the absence of protein S. A detailed characterization of the structure and function of this protein is unfortunately not available, and elucidation of the relationship between the bovine protein S binding protein and the putative APC cofactor must await purification of both components and characterization of their structural and functional properties. Second, the new APC cofactor appears to be involved in APC-mediated degradation of both factors VIII and V, as the APC resistance is less pronounced in the factor Xa assay than in the APTT- and factor IXa-based assays. Third, the results of mixing experiments (Fig. 4) suggested the anticoagulant response in the APC-APTT method to be exponential. This probably explains the skewed distribution of APC-dependent prolongations of clotting time observed among healthy controls.

Several of the coagulation factors were originally postulated on the basis of observations made in patients with bleeding problems—e.g., factors V and VIII. The coagulation factor that corrected the bleeding problem was purified many years after the original clinical observation. The proteins involved in the protein C anticoagulant system were identified via a different route. Proteins C and S were purified as vitamin K-dependent proteins with unknown functions, and their anticoagulant properties were delineated after a few years (1, 3). Immunological assays were then designed and used to investigate individuals with thromboembolic disease, as deficiencies of these proteins would hypothetically lead to a thrombotic tendency. The association between thromboembolic disease and deficiency of proteins C or S is now well established (1, 3, 4). The now hypothesized cofactor to APC is the first anticoagulant factor to be proposed on the basis of observations made in an individual patient with thromboembolic disease.

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1. Esmon, C. T. (1989) *J. Biol. Chem.* **264,** 4743–4746.
2. Kane, W. H. & Davie, E. W. (1988) *Blood* **71,** 539–555.
3. Dahlbäck, B. (1991) *Thromb. Haemostasis* **66,** 49–61.
4. Malm, J., Laurell, M., Nilsson, I. M. & Dahlbäck, B. (1992) *Thromb. Haemostasis* **68,** 7–13.
5. Dahlbäck, B. (1986) *J. Biol. Chem.* **261,** 12022–12027.
6. Dahlbäck, B., Nilsson, I. M. & Frohm, B. (1983) *Blood* **62,** 218–225.
7. Bell, G. I., Karam, J. H. & Rutter, W. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 5759–5763.
8. Kogan, S. C., Doherty, M. & Gitschier, J. (1987) *N. Engl. J. Med.* **317,** 985–990.
9. Donnér, M., Holmberg, L., Kristoffersson, A.-C. & Nilsson, I. M. (1991) *Br. J. Haematol.* **78,** 403–407.
10. Conney, K. A., Nichols, W. C., Bruch, M. E., Bahou, W. F., Shapiro, A. D., Bowie, E. J. W., Gralnick, H. R. & Ginsburg, D. (1991) *J. Clin. Invest.* **87,** 1227–1233.
11. Peak, I. R., Bowen, D., Bignell, P., Liddell, M. B., Sadler, J. E., Standen, G. & Bloom, A. L. (1990) *Blood* **76,** 555–561.
12. Mitchell, C. A., Rowell, J. A., Hau, L., Young, J. P. & Salem, H. H. (1987) *N. Engl. J. Med.* **317,** 1638–1642.
13. Dahlbäck, B., Hildebrand, B. & Malm, J. (1990) *J. Biol. Chem.* **265,** 8127–8135.
14. Suzuki, K., Deyashiki, Y., Nishioka, J. & Toma, K. (1989) *Thromb. Haemostasis* **61,** 337–342.
15. Owen, M. C., Brennan, S. O., Lewis, J. H. & Carrell, R. W. (1983) *N. Engl. J. Med.* **309,** 694–698.
16. Walker, F. J. (1986) *J. Biol. Chem.* **261,** 10941–10944.